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Essential oil from *Saussurea costus* inhibits proliferation and migration of Eca109 cells *via* mitochondrial apoptosis and STAT3 signaling

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ABSTRACT

Objective: To investigate the effect and its underlying molecular mechanisms of essential oil from *Saussurea costus* in esophageal cancer cell line Eca109.

Methods: The chemical composition of essential oil from *Saussurea costus* was investigated by gas chromatography-mass spectrometry (GC-MS). The anti-proliferative, anti-migrative, and apoptotic effects of essential oil from *Saussurea costus* against Eca109 cells were analyzed. Moreover, the expression of proteins associated with cell cycle, metastasis, and apoptosis was determined.

Results: GC-MS analysis showed that essential oil from *Saussurea costus* was predominantly comprised of sesquiterpenes. *Saussurea costus* essential oil inhibited the viability of Eca109 cells in a dose-and time-dependent manner with IC_{50} values of (24.29±1.49), (19.16±2.27) and (6.97±0.86) μg/mL at 12, 24, and 48 h, respectively. The expression levels of target proteins in the cell cycle (phase G_1/S), including cyclin D1, p21, and p53, were affected by *Saussurea costus* essential oil. The essential oil also downregulated the expression of metastasis-related proteins MMP-9 and MMP-2. Moreover, it induced apoptosis of Eca109 cells through the mitochondrial pathway, as well as inhibition of STAT3 phosphorylation.

Conclusions: The essential oil from *Saussurea costus* exhibited anti-proliferative, anti-migrative, and apoptotic effects on Eca109 cells, and could be further explored as a potential anti-esophageal cancer agent.

KEYWORDS: *Saussurea costus*; Essential oil; Gas chromatography-mass spectrometry; Apoptosis; Mitochondrial pathway; STAT3; Esophageal cancer; Anti-proliferation; Anti-migration; Eca109

1. Introduction

Esophageal cancer is the eighth most common cancer in the world, with an estimated 572 000 newly diagnosed cases and over 509 000 deaths each year[1]. Surgical resection combined with chemoradiotherapy is a generally recommended strategy for the treatment of esophageal cancer[2]. The 5-year survival rate of patients with esophageal cancer is 15%-20% worldwide[3]. The exploration of the pathogenesis of esophageal cancer is important for improving its treatment and prognosis.

Traditional Chinese medicine is a potential source of anti-

Significance

It has been reported that essential oil from *Saussurea costus* has several pharmacological activities, such as immunomodulatory and anti-inflammatory activities. However, the anti-esophageal cancer effect of essential oil from *Saussurea costus* has not been clarified. Our study found that essential oil from *Saussurea costus* can induce the apoptosis of Eca109 cells. The underlying mechanism may be related to mitochondrial apoptosis and STAT3 signaling pathway.

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esophageal cancer therapies, which has been used by various researchers to search for novel anticancer drugs[4–6]. For example, vinca alkaloids and taxanes isolated from traditional Chinese medicines have been developed as clinical anticancer drugs[7,8].

Saussurea costus (*S. costus*), a well-known species belonging to the family Asteraceae, is a medicinal and aromatic plant[9]. *S. costus* has been used to treat various ailments including stomach ulcers, asthma, and bronchitis[10,11]. As *S. costus* has a variety of pharmacological effects, it has attracted great attention from researchers. The main chemical components of *S. costus* are monoterpenes and sesquiterpenes. It has been reported that the essential oils with costunolide and dehydrocostone as the main components have the most significant inhibitory effect on the proliferation of cancer cells[12,13]. However, the effects of *S. costus* essential oil on Eca109 cells have been rarely reported. Therefore, the purpose of the present study was to investigate the anti-esophageal cancer effects of essential oil from *S. costus* and its underlying molecular mechanisms.

2. Materials and methods

2.1. Preparation of essential oil from S. costus

S. costus (Falc.) Lipech (Family Compositae) roots were collected in Wufeng County, Hubei Province, China in July 2015 and identified by Prof. Dingrong Wan (School of Pharmacy, South Central University for Nationalities, Wuhan, China). A voucher specimen (no. SC0187) was deposited in School of Pharmacy, South Central University for Nationalities. The air-dried *S. costus* roots (4.5 kg) were mechanically ground and then extracted by hydrodistillation using the Clevenger-type device (material-to-water ratio, 2:11). After 12 h of distillation, the essential oil from *S. costus* (18.5 mL) was collected, dried over anhydrous sodium sulfate, and stored at −80 ℃ for further analysis.

2.2. GC–MS analysis of essential oil from S. costus

The volatile components in essential oil from *S. costus* were analyzed using an Agilent 7890A gas chromatograph (Agilent Technologies, Inc.) with an HP-5MS 5% phenylmethyl siloxane capillary column (30.00 m × 0.25 mm, 0.25 μm film thickness) coupled to an Agilent 5975C mass selective detector. Twenty μL of essential oil from *S. costus* was dissolved in 1.0 mL hexane, and then the solution was dried over anhydrous sodium sulfate. The supernatant was filtered and stocked at 4 ℃ until use. Samples (0.1 μL) were injected in split mode (60:1 ratio). Helium (99.999%) was used as carrier gas with a flow rate of 2 mL/min. The oven temperature was initially held at 60 ℃ for 3 min and then increased to 260 ℃ at a rate of 4 ℃/min. The injector and detector temperatures were set to 250 ℃ and 280 ℃, respectively. Quantitative data was obtained using percent peak area. The working parameters of EI-MS were: ionization voltage, 70 eV; ion source temperature, 200 ℃. The collected mass spectra (from chromatographic peaks) were compared with reference spectra from the NIST08 database to identify oil components.

2.3. Cell culture

Eca109 cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium (HyClone; Cytiva) with 10% fetal bovine serum (Tianhang), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 at 37 ℃. The medium was changed every 2 d. Experiments were performed using log-phase cells.

2.4. MTT assay

MTT assays were used to detect the effect of essential oil from *S. costus* on the viability of Eca109 cells. Eca109 cells were inoculated in 96-well plates (1×10^5 cells/well). Stock solutions were obtained by dissolving essential oil from *S. costus* in dimethyl sulfoxide before use. Eca109 cells were treated with essential oil from *S. costus* at different concentrations (0, 5, 10, 15, 20, 25 and 30 μg/mL) for 12, 24, and 48 h. The formazan was solubilized using dimethyl sulfoxide, and then the optical density (OD) was read at 562 nm by a microplate reader (Bio-Rad Laboratories, Inc.).

The inhibition rate was calculated according to the following formula: Inhibition rate (%) = $[1 - (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})] \times 100$[14,15], where OD_{sample} is OD value of Eca109 cells after adding essential oil from *S. costus*; OD_{blank} is OD value without adding both essential oil from *S. costus* and Eca109 cells; $OD_{control}$ is OD value of Eca109 cells without adding essential oil from *S. costus*. The IC_{50} value of essential oil from *S. costus* was calculated using GraphPad Prism software version 7.0 (GraphPad Software, Inc.).

2.5. Wound healing assay

Eca109 cells (1×10^5 cells/well) were inoculated in 6-well plates with different concentrations of essential oil from *S. costus*. Then, a scratch-wound healing assay was performed. Linear scratch wounds were made in the cell monolayers with a 10-μL pipette tip, and the images of scratch wounds were captured using a phase-contrast microscope (magnification: ×20; Leica Microsystems GmbH) in the same area at 0, 6, and 12 h after incubation with serum-free Dulbecco's Modified Eagle Medium. The wound surface area was calculated using ImageJ software (Version 5.0; National Institutes of Health).

2.6. Observation of morphological changes

Eca109 cells (1×10^5 cells/well) were cultured in 6-well plates and

incubated for 24 h at 37℃ with essential oil from *S. costus* (0, 7.5, 15, or 22.5 μg/mL) or cisplatin (10 μg/mL; Sinopharm Chemical Reagent Co., Ltd.). After incubation, the cellular morphological changes of Eca109 cells were observed and photographed at a magnification of ×20 under a phase-contrast microscope (Leica Microsystems GmbH). The Eca109 cells were subsequently immobilized with cell fixative (Acetic acid: methanol =1:3) at room temperature for 3-5 min and stained for 5 min with Hoechst 33258 (10 μg/mL) at room temperature in the dark. Subsequently, the cellular morphological changes of the nuclei were observed and photographed at a magnification of ×40 under a phase-contrast microscope (Leica Microsystems GmbH).

2.7. Flow cytometry analysis

The effect of the essential oil from *S. costus* on apoptosis of Eca109 cells was determined using the annexin V-FITC kit (Beyotime Institute of Biotechnology). After incubation with essential oils from *S. costus* (7.5, 15, or 22.5 μg/mL) for 24 h, Eca109 cells were collected and resuspended in a binding buffer. Then, annexin V-FITC and propidium iodide (PI) were added and incubated in the dark at room temperature for 15 min before being analyzed by flow cytometry (BD Biosciences).

2.8. Annexin V–FITC apoptosis detection assay

The cells were collected after apoptosis induction and then centrifuged at 1000 ×*g* for 5 min followed by resuspension in 195 μL of annexin V-FITC binding solution. Subsequently, 5 μL of annexin V-FITC and 10 μL of PI staining solution were added, mixed gently, and incubated for 10-20 min at room temperature in darkness. Cells were then observed under a fluorescence microscope (magnification: ×40; OLYMPUS FV1000, FV10-ASW 4.1).

2.9. Western blotting assay

Eca109 cells were incubated with different concentrations of the essential oil from *S. costus* (0, 7.5, 15, 22.5 μg/mL) for 24 h. Cells were then collected and lysed with a lysis buffer supplemented with protease inhibitors (Beyotime Institute of Biotechnology). The protein concentrations were determined with a BCA kit. Cell lysates were denatured in boiling water, separated (15 μg per lane) *via* 7.5%-12.5% SDS-PAGE, and electrotransferred on PVDF membranes. After the transfer, 5% skim milk was used to block membranes for 1 h at room temperature, and then the membranes were incubated with the following primary antibodies (Cell Signaling Technology, Inc.; 1:1000) at 4℃ for 12 h: Bax (cat. no. #5023), Bcl-2 (cat. no. #15071), cleaved caspase-3 (cat. no. #9661), cleaved caspase-9 (cat. no. #9508), PARP (cat. no. #9542), MMP-2 (cat. no. #40994), MMP-9 (cat. no. #13667), STAT3 (cat. no. #12640), phosphorylated (p)-STAT3 (Tyr705) (cat. no. #9131), cyclin D1 (cat. no. #2978), p53 (cat. no. #2524), p21 Waf1/Cip1 (cat. no. 2947) and β-actin (cat. no. 3700; 1:2000). The membranes were further incubated with HRP-conjugated goat anti-mouse IgG3 (cat. no. #75952; 1:4000) and HRP-mouse anti-rabbit (cat. no. #3678; 1:2000) secondary antibodies (Cell Signaling Technology, Inc.) at room temperature for 1 h. After incubation, the membranes were washed three times with TBS-Tween 20 (0.1%), and the protein expression was determined with an enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Quantity One and Image Lab Version 4.0 (Bio-RAD Laboratories, Inc.) softwares were used for density determination.

2.10. Statistical analysis

All experimental data were expressed as mean ± SD of at least three independent experiments. GraphPad Prism 6.0 software was used for statistical analysis. Statistical comparisons were performed using one-way analysis of variance and Dunnett's *post hoc* test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Chemical analysis of essential oil from S. costus

The essential oil of *S. costus* had a yield of 0.35% (*w/w*) based on its dry weight. Thirteen different compounds including seven sesquiterpenes were identified from the essential oil from *S. costus* by GC-MS analysis, representing 95.84% of the total oil. Their relative abundance and retention times are presented in Figure 1 and Supplementary Table 1. The essential oil from *S. costus* was mainly comprised of 1,8-cyclopentadecadiyne (41.60%), 3a,5,6,7,8,8a,9,9a-octahydro-4,8a-dimethyl-6-(1-methylethenyl)-naphtho[2,3-*d*]-1,3-dioxol-2-one (16.70%) and oxabicyclo[4.1.0]heptane (13.80%), followed by β-guaiene (5.06%), 3-ethyl-3-hydroxy-androstan-17-one (5.02%), *cis*-4,7,10,13,16,19-docosahexaenoic acid (4.03%), 2,4a,5,6,9,9a-hexahydro-3,5,5,9-tetramethyl-1*H*-benzocycloheptene (3.40%), oxacyclotetradeca-4-11-diyne (2.61%), 1,11-hexadecadiyne (0.91%), γ-ionone (0.85%), elemene (0.83%), sativene (0.52%) and isolongifolene (0.51%).

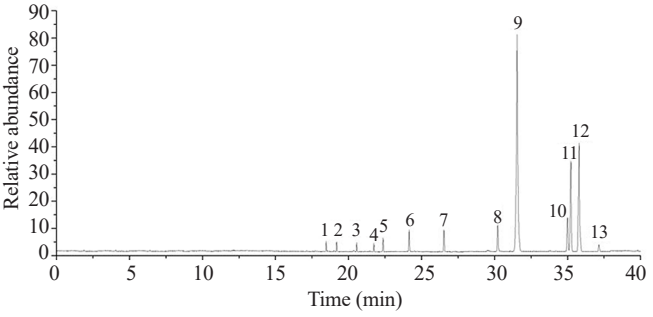

Figure 1. Gas chromatography-mass spectrometry analysis of essential oil from *Saussurea costus*.

3.2. Effect of essential oils from S. costus on Eca109 cell viability

To study the effect of essential oil from *S. costus* on the viability of Eca109 cells, Eca109 cells were incubated with different concentrations of essential oil from *S. costus* for 12, 24, and 48 h. As presented in Figure 2A, the essential oil from *S. costus* inhibited Eca109 cell viability in a dose- and time-dependent manner. The IC_{50} values of Eca109 cells at 12, 24, and 48 h were (24.29±1.49), (19.16±2.27), and (6.97±0.86) μg/mL, respectively. Western blotting analysis showed that the expression levels of typical anti-proliferation proteins p53 and p21 Waf1/Cip1 were significantly increased (P<0.05), while the expression of cyclin D1 was significantly decreased (P<0.05) in Eca109 cells after incubation with essential oil from *S. costus* (15 μg/mL) for 24 h (Figure 2B and C). These results suggested that essential oil from *S. costus* inhibited the proliferation of Eca109 cells by regulating key proteins in cell cycle.

3.3. Effect of essential oil from S. costus on migration of Eca109 cells

Wound healing assay was used to examine the effect of essential oil from *S. costus* on migration of Eca109 cells. In preliminary experiments, we observed that essential oil from *S. costus* (at high concentration levels over 10 μg/mL) strongly induced apoptosis, and inhibited cell migration. Therefore, 10 μg/mL of *S. costus* essential oil was a suitable choice to verify migration ability of Eca109 cells in further experiment. As shown in Figure 3A, the migration of Eca109 cells was significantly inhibited by treatment with essential oil from *S. costus*. In Western blotting assay, the expression of both MMP-2 and MMP-9 was markedly decreased in Eca109 cells treated with essential oil from *S. costus* (P<0.05) (Figure 3B). Therefore, essential oil from *S. costus* can inhibit the migration of Eca109 cells.

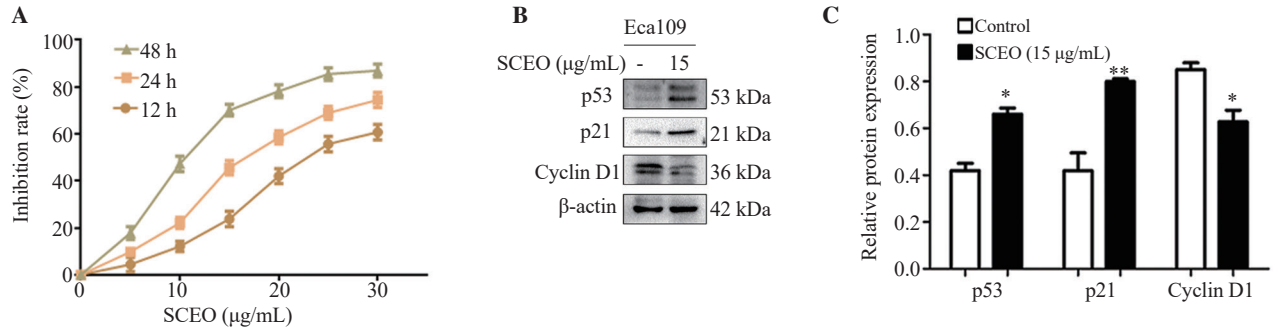

Figure 2. Effect of *Saussurea costus* essential oil (SCEO) on Eca109 cell viability. (A) Time- and dose-dependent effects of SCEO on Eca109 cells. (B) Effects of SCEO on the expression of p53, p21, and cyclin D1 protein by Western blotting assay. (C) Quantification of proliferation-associated protein expression. Data are presented as mean ± SD of at least three independent experiments. $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control.

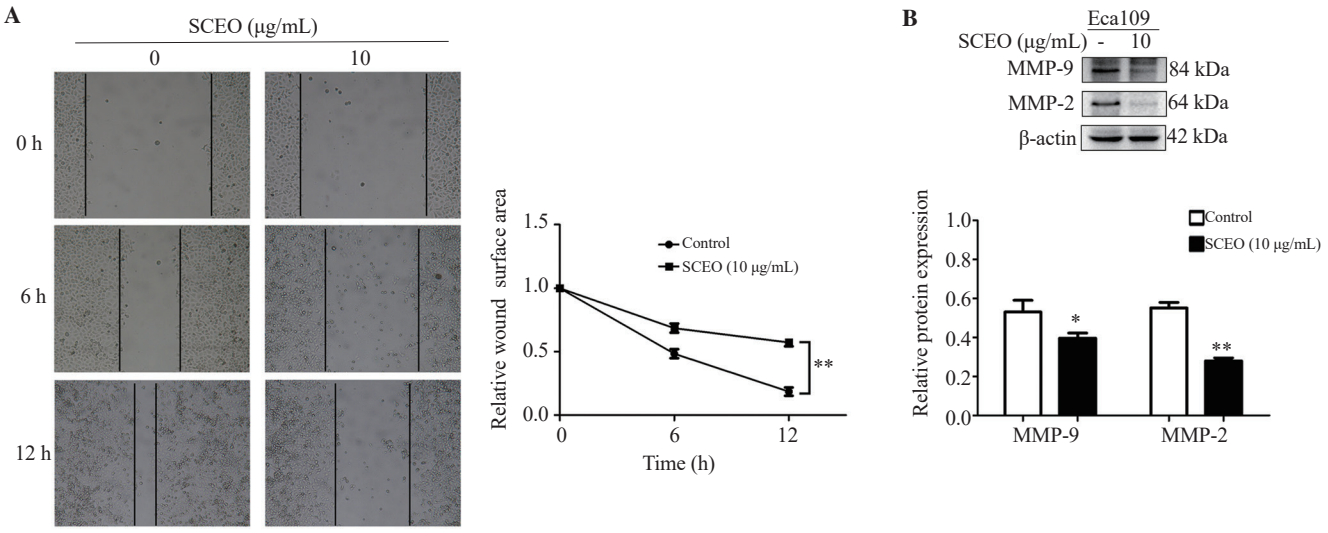

Figure 3. SCEO inhibits the migration of Eca109 cells. (A) Wound healing assay was performed. After the cells were treated with SCEO (10 μg/mL) for 0, 6, and 12 h, photographs were captured under a microscope. (B) Western blotting analysis of MMP-9 and MMP-2 in Eca109 cells treated with SCEO. Data are presented as mean ± SD of at least three independent experiments. $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control.

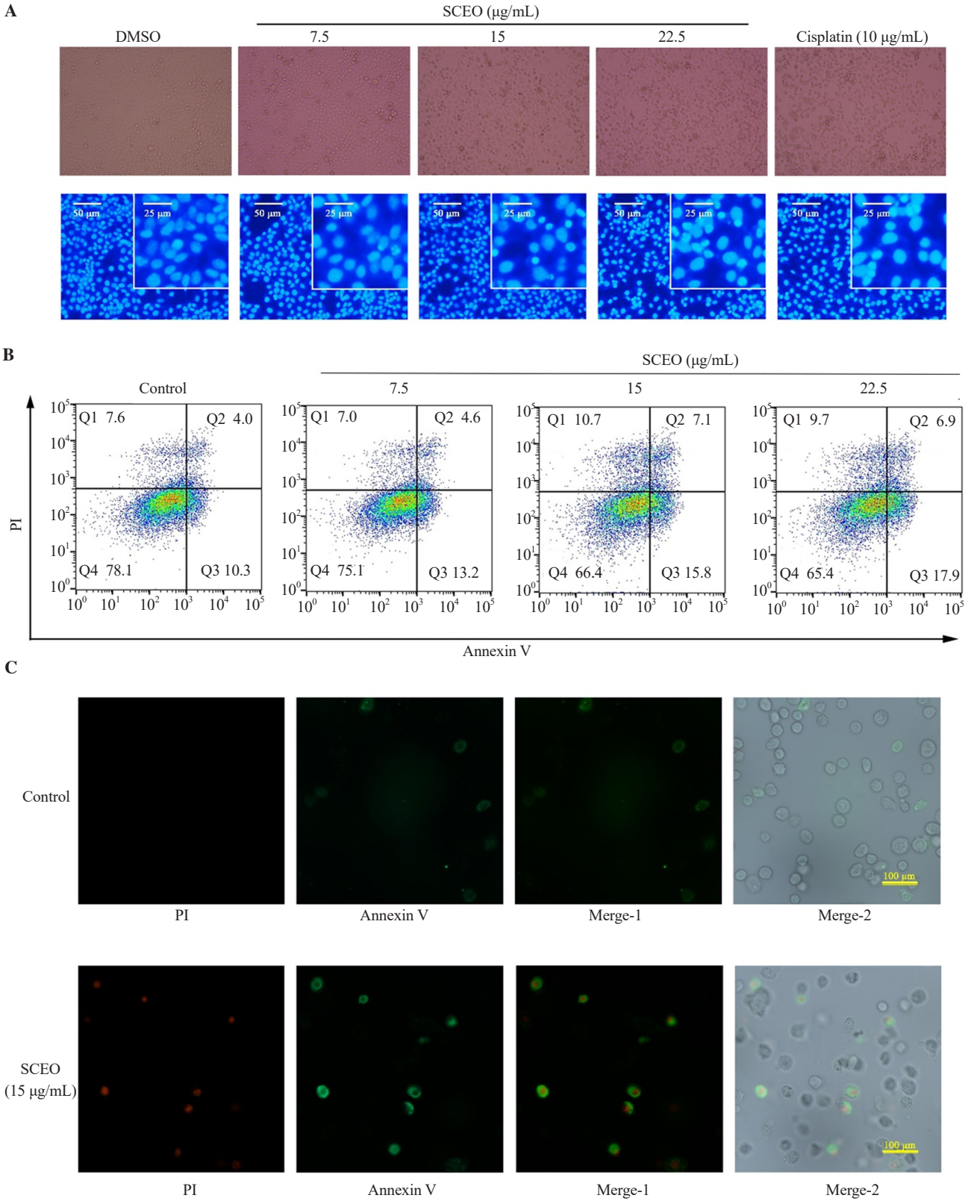

Figure 4. SCEO induces Eca109 cell apoptosis. (A) Morphological analysis of Eca109 cells treated with SCEO. (B) Eca109 cells were treated with 7.5, 15, and 22.5 μg/mL of SCEO for 24 h. Apoptosis was assessed *via* flow cytometry after annexin V-FITC/propidium iodide (PI) staining. (C) Eca109 cells were treated with SCEO for 24 h, stained with annexin V-FITC/PI, and observed under a fluorescence microscope.

3.4. Effect of essential oil from S. costus on Eca109 cell apoptosis

Morphological changes of Eca109 cells treated with essential oil from *S. costus* and cisplatin were observed. As presented in Figure 4, after treatment with essential oil from *S. costus*, Eca109 cells showed some typical apoptotic morphological characteristics including apoptotic corpuscles, cell shrinkage, and floating, while the cells in the control group showed no significant changes. After Hoechst 33258 staining, Eca109 cells in the control group were uniformly

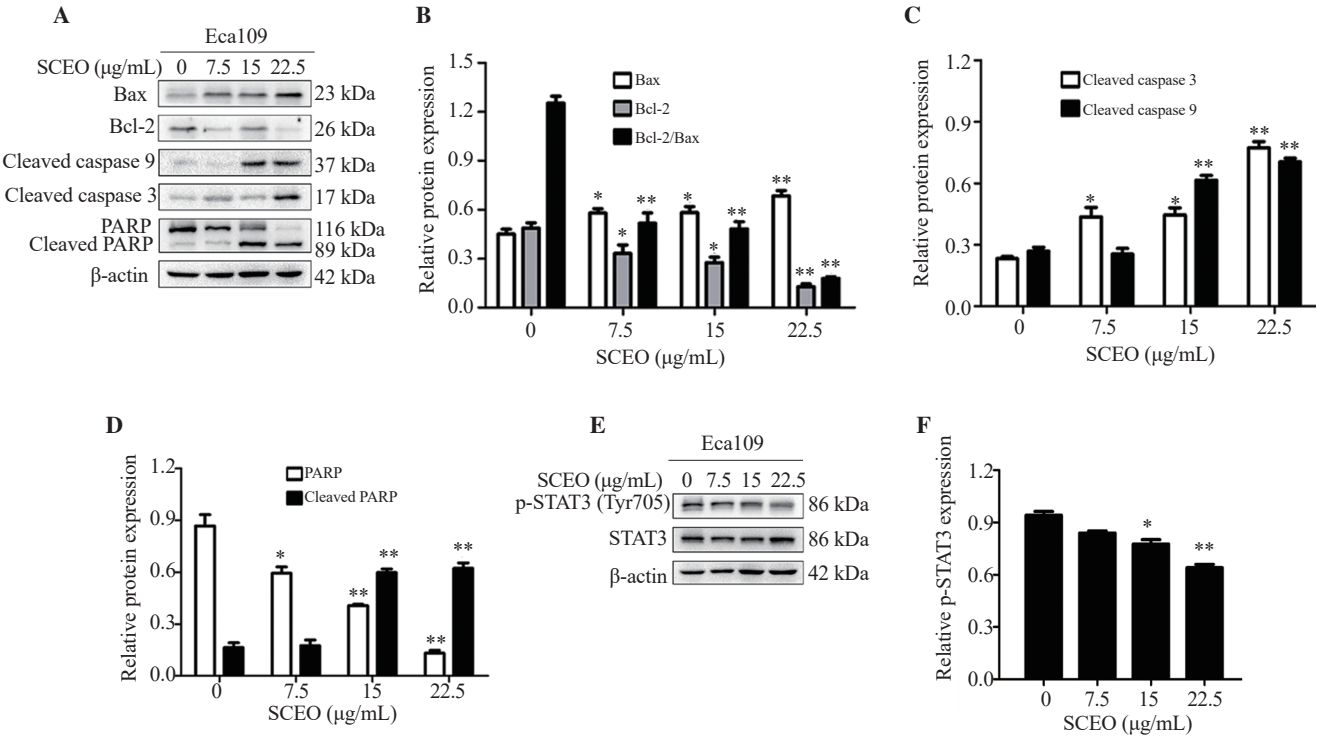

Figure 5. SCEO activates mitochondrial apoptotic pathways and inhibits STAT3 phosphorylation in Eca109 cells. (A) Western blotting analysis of apoptosis-associated proteins in Eca109 cells treated with SCEO. (B-D) Relative levels of Bax, Bcl-2, Bcl-2/Bax ratio, cleaved caspase-3, cleaved caspase-9, PARP, and cleaved PARP. (E) Western blotting analysis of p-STAT3 and STAT3 in Eca109 cells treated with SCEO. (F) Relative levels of p-STAT3 (Tyr705) proteins. Data are presented as mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* control.

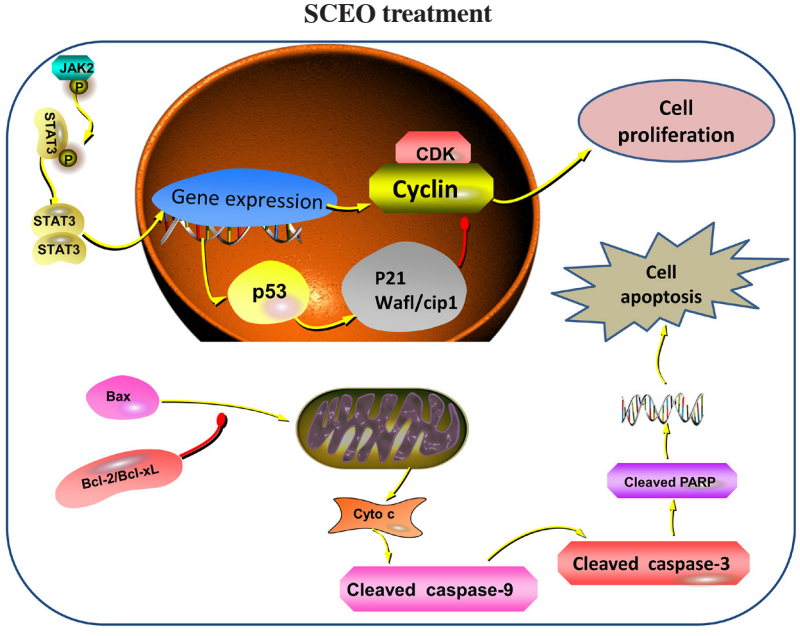

Figure 6. Schematic summary of the proposed underlying mechanisms of SCEO-induced Eca109 cell apoptosis. cyto c: cytochrome c.

stained and apoptotic bodies were rarely seen. In contrast, *S. costus* essential oil-treated Eca109 cells exhibited apoptotic morphology with enhanced nuclear fluorescence intensity and an increased number of apoptotic corpuscles. The essential oil from *S. costus* induced apoptosis in Eca109 cells in a dose-dependent manner. In addition, the apoptotic rate was assessed by annexin-V/PI double staining and flow cytometry analysis. The *S. costus* essential oil-treated Eca109 cells had a higher apoptotic rate than that of the untreated group with 17.8%, 22.9%, and 24.8% at 7.5, 15, and 22.5 μg/mL, respectively.

3.5. Essential oil from S. costus activates mitochondrial apoptosis and suppresses STAT3 signaling in Eca109 cells

The expressions of proteins related to apoptosis were detected by Western blotting assay (Figure 5). The essential oil from *S. costus* significantly increased the expression of cleaved caspase-9, cleaved caspase-3, and cleaved PARP, and reduced the ratio of Bcl-2/Bax in a dose-dependent manner. The results suggested that the activation of the mitochondrial apoptosis pathway may be involved in the apoptosis of Eca109 cells induced by essential oil from *S. costus*.

As presented in Figure 5, essential oil from *S. costus* dose-dependently inhibited the phosphorylation of STAT3 at Tyr705 but did not inhibit the total expression of STAT3 protein. Figure 6 shows the molecular mechanism of *S. costus* essential oil-induced apoptosis of Eca109 cells.

4. Discussion

S. costus is a perennial Chinese folk medicinal plant. However, the pharmacological effects and the underlying molecular mechanisms of *S. costus* in human esophageal squamous cell carcinoma are still poorly understood. A large number of terpenoids contained in essential oils have been reported to possess anticancer properties[16,17]. In the present study, the chemical composition of essential oil from *S. costus* was analyzed by GC-MS and it was found that it consisted mainly of sesquiterpenes which might be responsible for its anticancer activity observed. The results of MTT assay showed that the inhibition of essential oil from *S. costus* on Eca109 was time- and dose-dependent. Moreover, Western blotting assay showed that essential oil from *S. costus* inhibited cell viability by impeding the cell cycle of Eca109. MMPs play an important role in the migration and invasion of tumor cells[18]. The essential oil from *S. costus* also demonstrated significant inhibitory effects on Eca109 cell migration by downregulating the expression of MMP-9 and MMP-2.

Cell death takes many forms, of which necrosis and apoptosis are the two most common types[19]. Necrosis represents passive cell death resulting from external stimulation, while apoptosis is an active process of programmed cell death regulated by genes[20]. In the present study, we found that the morphology of Eca109 cells was significantly altered after treatment with essential oil from *S. costus*, including concentrated chromatin, enhanced fluorescence, and bright blue spots. Annexin-V/PI double staining and flow cytometry analysis showed that essential oil from *S. costus* could induce the apoptosis of Eca109 cells.

Caspase-3 occupies a central role in mediating apoptosis, serving as a key downstream executor of apoptosis. Bcl-2 and Bax are homodimeric proteins that counteract each other. Increased Bcl-2 protein content results in increased heterodimer formation with Bax, thus inhibiting apoptosis[21]. During apoptosis, pro-apoptotic members of the Bcl-2 family are activated and transferred to mitochondria *via* dephosphorylation or caspase-mediated protein hydrolysis. Bax induces mitochondrial release of proteins, including whole cytochrome c. Only whole cytochrome c can induce caspase activation. Bax can directly bind to apoptotic protease activating factor-1, causing it to oligomerize and recruit caspase-9 enzymes through the corresponding caspase recruitment domains to form an apoptotic complex, which in turn activates caspase-3 and initiates the caspase cascade reaction to induce apoptosis. Pro-apoptotic Bax and anti-apoptotic Bcl-2 regulate intrinsic apoptotic pathways[22]. Caspase-9 and caspase-3 serve as target proteins during apoptosis, and their precursor forms are cleaved during activation[23]. Poly ADP-ribose polymerase (PARP) acts as a target protein of caspase-3 and affects the repair of cancer cells.

This study also investigated whether essential oil from *S. costus* could induce apoptosis in Eca109 cells through an internal apoptosis pathway. Western blotting results showed that the expressions of cleaved caspase-9, cleaved caspase-3, and cleaved PARP were increased in a dose-dependent manner after cells were treated with essential oil from *S. costus*, while the ratio of Bcl-2/Bax was decreased. These results suggested that essential oil from *S. costus* induced the apoptosis of Eca109 cells through the mitochondrial apoptosis pathway.

It was hypothesized that essential oil from *S. costus* might affect a key target protein to trigger a series of downstream signals. The transcription factor STAT3 can regulate apoptosis in tumor cells as well as cell proliferation[24]. As the previous experiments observed that essential oil from *S. costus* exhibited anti-proliferative and apoptotic effects on Eca109 cells, STAT3 protein expression was analyzed. The phosphorylation of STAT3 in esophageal cancer is primarily mediated through phosphorylation of Tyr705[25]. STAT3 is a member of a family of transcription factors that regulate proliferation and apoptosis[26]. The level of STAT3 phosphorylation is closely associated with p53 which has DNA-binding activity and can be regulated by phosphorylation. When p-STAT3 expression is downregulated, STAT3-dependent gene transcriptional activity is inhibited. As the phosphorylation of STAT3 is regulated by multiple mechanisms, it can be mediated *via* different pathways and protein

kinases[27]. The results showed that essential oil from *S. costus* dose-dependently inhibited STAT3 phosphorylation, and upregulated the expression of p53, leading to p53-mediated apoptosis and transcription of various downstream genes. Inhibition of cell proliferation is associated with cell cycle arrest[28]. Therefore, the effects of essential oil from *S. costus* on cell cycle-associated proteins were analyzed. Cyclin D1 is a regulator of cell cycle progression; in contrast, p53 and p21 Waf1/Cip1 play a key role in negative regulation of cell cycle[29-31]. The p21 Waf1/Cip1 protein can bind to cyclin/cyclin-dependent kinase complexes to induce cell cycle arrest[32,33]. We found that the essential oil from *S. costus* increased the expression level of p21 Waf1/Cip1. All these results suggested that the essential oil from *S. costus* has the potential to be a targeted chemotherapy agent for esophageal squamous cell carcinoma. However, the anti-esophageal cancer effects of essential oil from *S. costus* still need to be further verified in *in vivo* and clinical studies.

In conclusion, GC-MS analysis showed that the main components of essential oil from *S. costus* were sesquiterpenes. Also, *S. costus* essential oil can inhibit the expression of cyclin D1 which is essential for the G_1 to S phase transition, as well as the expression of MMP-9 and MMP-2, thereby inhibiting proliferation and migration of Eca109 cells. Moreover, it could induce apoptosis in Eca109 cells through the mitochondrial apoptosis pathway and inhibition of STAT3 phosphorylation.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

WQ, YXZ, and ZW planned and designed the research work. HXF, LWX, and ZR conducted the experiments and collected the data. CM and SR were involved in data analysis and interpretation. HXF, ZR, and WC wrote the manuscript. HXF, WC, and ZR revised the manuscript. HXF, LWX, ZR, ZW, WC, CM, WQ, SR, and YXZ finally approved the version to be published.

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